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TANGO1 Facilitates Cargo Loading at Endoplasmic Reticulum Exit Sites

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SUMMARY

A genome-wide screen revealed previously unidentified components required for transport and Golgi organization (TANGO). We now provide evidence that one of these proteins, TANGO1, is an integral membrane protein localized to endoplasmic reticulum (ER) exit sites, with a luminal SH3 domain and a cytoplasmic proline-rich domain (PRD). Knockdown of TANGO1 inhibits export of bulky collagen VII from the ER. The SH3 domain of TANGO1 binds to collagen VII; the PRD binds to the COPII coat subunits, Sec23/24. In this scenario, PRD binding to Sec23/24 subunits could stall COPII carrier biogenesis to permit the luminal domain of TANGO1 to guide SH3-bound cargo into a growing carrier. All cells except those of hematopoietic origin express TANGO1. We propose that TANGO1 exports other cargoes in cells that do not secrete collagen VII. However, TANGO1 does not enter the budding carrier, which represents a unique mechanism to load cargo into COPII carriers.

INTRODUCTION

Newly synthesized secretory proteins exit the endoplasmic reticulum (ER) in so-called COPII-coated vesicles (Bonifacino and Glick, 2004; Lee et al., 2004). A large number of proteins are required for the biogenesis of these vesicles, including chaperones and enzymes that fold the newly synthesized protein in the lumen of the ER, receptors that bind secretory cargo and help their recruitment into the newly forming carriers, structural coat proteins that help mold the flat ER membrane into a bud and promote its growth into a vesicular element, and components that catalyze the budding of cargo-loaded carriers from the ER (Herrmann et al., 1999; Lee et al., 2004).

COPII coat-mediated budding of vesicles from the ER is relatively well characterized. Two layers of coat proteins comprised of a Sec23/24 inner layer and a Sec13/31 outer layer are recruited to the membrane surface by Sar1-GTP at specific ER exit sites that are marked by the protein Sec16 (Bonifacino and Glick, 2004; Lee et al., 2004). Less is known about how cargo proteins are collected into nascent vesicles. Whether it is a passive process (bulk flow) or actively mediated by cargo receptors has been debated extensively (Wieland et al., 1987; Lee et al., 2004). Support in favor of the bulk flow proposal, however, is waning by recent growing evidence that cargo loading into COPII carriers utilizes receptors and chaperones (Lee et al., 2004). The receptors identified thus far appear to exit with the outgoing cargo, whereas the chaperones mostly stay behind in the ER (Lee et al., 2004).

Prominent examples of cargo receptors are ERGIC 53 for the transport of coagulation factors V and VIII, cathepsin C and Z, and α1-antitrypsin; Erv29p/Surf4 for the export of soluble cargo proteins such as precursor of α factor mating pheromone; and Emp24p for GPI-anchored membrane protein Gas1p (Belden and Barlowe, 2001; Muniz et al., 2000; Nichols et al., 1998; Nyfeler et al., 2008; Vollenweider et al., 1998). Additional relatives of these three proteins have also been identified (Baines and Zhang, 2007). Another well-characterized cargo receptor is the KDEL receptor, which traffics KDEL-containing proteins from the Golgi to the ER (Munro and Pelham, 1987; Pfeffer, 2007). Chaperones primarily assist in the folding of newly synthesized proteins and, therefore, are required for export from the ER. In addition to the conventional chaperones, BAP31 and the yeast-specific Shr3p also appear to function as cargo receptors (Baines and Zhang, 2007; Kota et al., 2007). It has been estimated that as many as 35% of the entire human genome enters the secretory pathway at the ER. Considering the quantity and the diversity of cargo exported by this compartment, the number of receptors identified thus far seems surprisingly low.

We performed a genome-wide screen in *Drosophila* tissue culture S2 cells to identify transport components (Bard et al., 2006). This screen revealed a number of previously unidentified genes required for transport and Golgi organization (TANGO

genes). TANGO1 from this collection emerges as a guide for loading the cargo molecule collagen VII into COPII carriers. Our findings provide further proof that collagen VII export from the ER is not by bulk flow.

RESULTS

TANGO1 Localizes to ER Exit Sites

Drosophila TANGO1 contains an SH3-like domain at its N terminus, followed by a potential transmembrane (TM) domain, a coiled-coiled domain, and a C-terminal, proline-rich domain (PRD). The mammalian ortholog of TANGO1 was identified by sequence comparison, cloned, and HA-epitope tagged at its C terminus. The domain organization of human TANGO1 is basically the same as *Drosophila* TANGO1 except for two closely situated potential transmembrane domains that were found in the databases (amino acids 1143–1165 and 1183–1205) (Figure 1A). A polyclonal antiserum was generated against the PRD (C-terminal amino acids 1884–1898) of TANGO1 and affinity purified. HA-tagged TANGO1 was expressed in COS7 cells and immunoprecipitated with anti-HA antibody and blotted with either anti-HA or anti-TANGO1 antibodies. Both antibodies recognized the exogenously expressed, tagged protein (Figure 1B, lanes 1 and 2). TANGO1 migrates slower than the expected molecular mass on SDS-PAGE. To detect the endogenous protein, HeLa cell extracts were either immunoprecipitated with anti-TANGO1 antibody followed by western blotting with anti-TANGO1 antibody (Figure 1B, lane 3), or cell extracts from HeLa (Figure 1B, lane 4) and A431 (Figure 1B, lane 5) were directly western blotted with anti-TANGO1 antibody. These experiments revealed a major polypeptide of > 250 kDa, which has the same mobility as the exogenously expressed, HA-tagged TANGO1. This indicates that we have cloned the mammalian TANGO1 and the antibody is suitable for further characterization of this polypeptide. It has been reported that the N-terminal 125 amino acid, which includes the SH3-like domain of TANGO1, is secreted (Bosserhoff et al., 2004). This portion has been given the name TANGO (the name is coincidently the same as our transport and Golgi organization) and MIA3 (melanoma inhibitory activity 3) (Bosserhoff et al., 2004). The reported sequence contains a termination codon at the 376 base pair region of TANGO1 and is listed as US patent WO 00/12762 (Bosserhoff et al., 2004); however, there are no such sequences in the NCBI databases. Our RT-PCR also did not reveal a termination codon at this site. Moreover, the database search for other species, including *Drosophila*, zebrafish, and mouse, did not reveal termination codons at the reported site. The origin of TANGO, therefore, remains speculative, but it is possible that, under certain conditions, a spliced variant of TANGO1 is secreted. However, our results here describe the full-length TANGO1 (1907 amino acids), which is localized to the ER exit sites and is not secreted (see below).

The affinity-purified antiserum was used to visualize the localization of endogenous TANGO1 protein in HeLa cells. The antibody staining localized to punctate structures, 80% of which colocalized with transfected Sec16L, a bona fide component of ER exit sites (Figure 1C, panel 1). The antibody also displayed a 60% colocalization with the endogenous ER exit site protein Sec31A (Figure 1C, panel 2). ERGIC-53 of the intermediate compartment (or ERGIC) appears as a collection of perinuclear elements and punctate elements that are distributed throughout the cytoplasm. There was only partial colocalization (30%) between TANGO1 and the dispersed (peripheral) ERGIC-53 (Figure 1C, panel 3). No colocalization was observed between TANGO1 and the perinuclear ERGIC-53 and between TANGO1 and β-COP (Figure 1C, panels 3 and 4). Thus, TANGO1 is localized to the majority of ER exit sites.

HeLa cells were transfected with control or TANGO1-specific siRNA. Immunofluorescence microscopy and western blotting with anti-TANGO1 antibody was used to determine the depletion of endogenous TANGO1. Immunofluorescence microscopy revealed the loss of TANGO1 staining from the ER exit sites (Figure S1A available online). Western blotting revealed > 90% reduction in TANGO1 protein levels (Figure S1B). Thus, TANGO1 localizes to the ER exit sites, the anti-TANGO1 antibody is suitable for analysis of the endogenous protein, and the siRNA efficiently depletes TANGO1 in mammalian cells.

The Topology of TANGO1 at ER Exit Sites

To determine the topology of TANGO1, we made use of the fact that digitonin can be used to selectively permeabilize the plasma membrane, and, after paraformaldehyde (PFA) fixation, treatment of the same cells with Triton X-100 permeabilizes both plasma membrane and intracellular membranes. The cytoplasmically oriented region of the protein should be visible by fluorescence microscopy in cells treated with digitonin, whereas detection of the luminal domain of a membrane protein should require treatment with Triton X-100. As shown in Figure 2A, an antibody that recognizes the luminal domain of ERGIC-53 required Triton permeabilization for binding to its cognate epitope. In contrast, GM130, which is peripherally attached to the cytoplasmic surface of *cis*-Golgi cisternae, was visible by immunofluorescence microscopy in cells treated with digitonin only (Figure 2A).

The anti-TANGO1 C-terminal-specific antibody was, therefore, used to reveal the orientation of this domain across the ER. As shown in Figure 2A, digitonin treatment followed by fixation with PFA was sufficient to visualize endogenous TANGO1 protein with the C-terminal specific, anti-TANGO1 antibody. This suggests that the C-terminal residues face the cytoplasmic side. When TANGO1 is expressed exogenously in cells and fixed with PFA, it yields a more diffused ER staining, but this should not affect the topology of the protein. To determine the orientation of the N-terminal SH3 domain, we generated a tagged version of TANGO1 in which a FLAG tag was placed between the signal sequence and the SH3 domain and an HA tag was introduced at the C terminus of TANGO1 (FLAG-TANGO1-HA). The transfected HeLa cells were treated with digitonin followed by fixation and incubated with either anti-FLAG or HA antibodies (stage I). The cells were then washed, treated with Triton X-100, and incubated with anti-TANGO1 antibody (stage II). The transfected cells were identified by higher TANGO1 staining compared with the nontransfected cells. These cells were also visualized for the staining with anti-FLAG or HA antibodies. Although HA antibody gave a strong signal in these cells (Figure 2B, lane 2), the N-terminally localized FLAG epitope was not detected in transfected

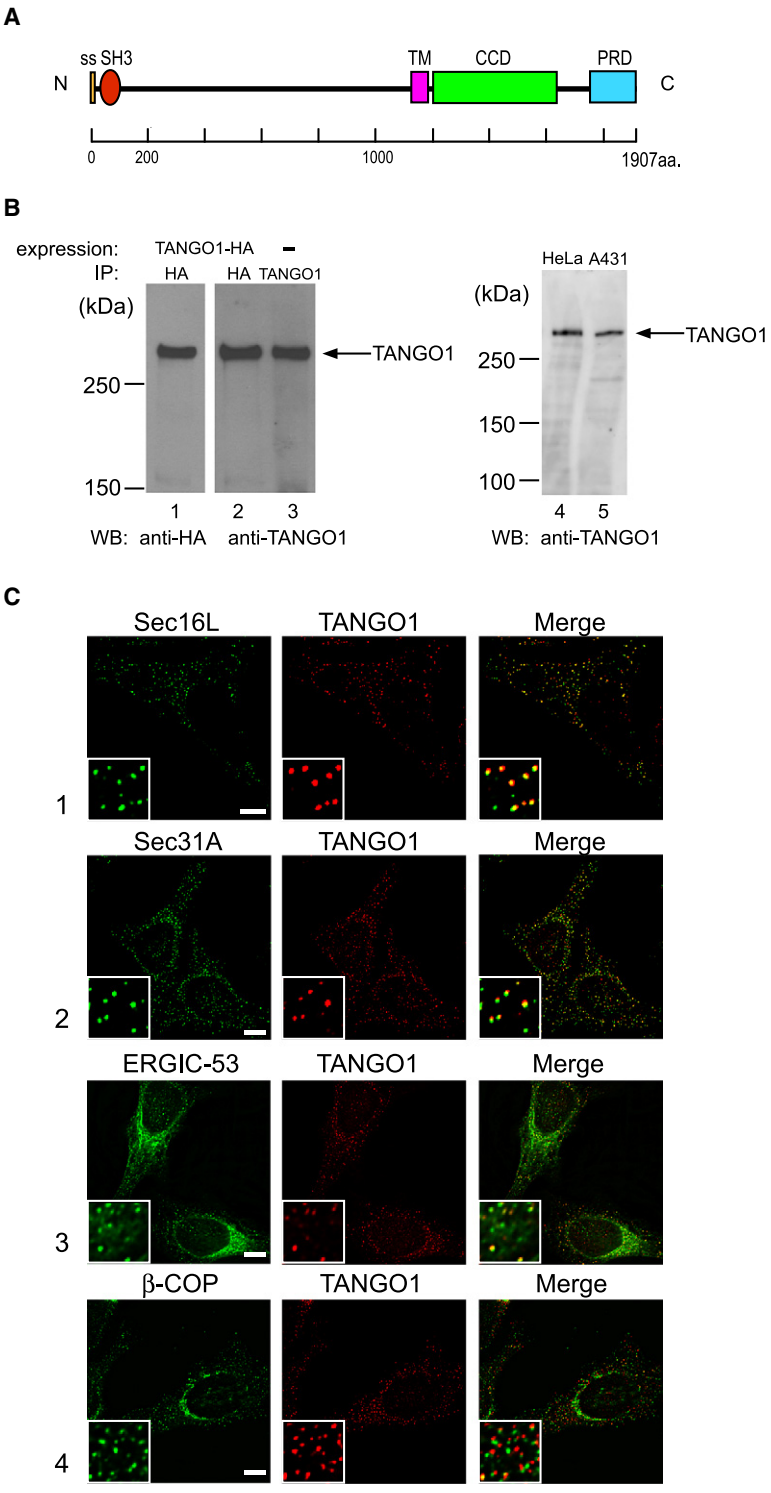

Figure 1. Cloning of Human TANGO1 and Its Localization

(A) Schematic representation of human TANGO1 domain organization. ss, signal sequence. SH3, Src homology 3-like domain. TM, putative transmembrane domain. CCD, coiled-coiled domain. PRD, proline-rich domain.

(B) TANGO1-HA was immunoprecipitated from transfected COS7 cells (lanes 1 and 2) and western blotted with anti-HA (lane 1) and anti-TANGO1 (lane 2) antibody, respectively. HeLa cell lysate was immunoprecipitated with anti-TANGO1 antibody and western blotted with anti-TANGO1 antibody (lane 3). HeLa (lane 4) and A431 (lane 5) cell lysates were western blotted with anti-TANGO1 antibody.

(C) Sec16L-GFP-transfected HeLa cells were stained with TANGO1 antibody. Potential colocalization of TANGO1 with Sec31A, ERGIC-53, and β-COP was tested with specific antibodies in HeLa cells. Images were deconvolved with Autoquant. Scale bar, 10 μm.

cells permeabilized with digitonin only (Figure 2B, lane 1). Treatment of these cells with Triton revealed both the N terminus FLAG and the C terminus HA tag as expected (Figure 2B, lanes 3 and 4). Thus, the transfected protein appears to show the correct topology, and the FLAG epitope is not accessible to the antibody in digitonin-permeabilized cells. This provides strong evidence that the SH3 domain is localized in the lumen of the ER.

As an additional and more quantitative approach to determine the orientation of TANGO1 in the ER, we tested the susceptibility of FLAG-TANGO1-HA to partial proteolysis with trypsin. FLAG-TANGO1-HA was expressed in COS7 cells; the cells were permeabilized with digitonin and then treated with trypsin. Following trypsin inactivation, the cells were lysed, and the lysates were immunoprecipitated with anti-FLAG antibody and western blotted with anti-FLAG antibody (Figure S2, top panel) or immunoprecipitated with anti-HA antibody followed by western blotting with anti-HA antibody (Figure S2, bottom panel). Under these conditions, the C-terminal HA tag was lost upon trypsinization. The N-terminal FLAG tag was, however, protected from proteolysis, and the corresponding TANGO1 protein was migrated with the mobility predicted for the remaining N terminus up to the two putative transmembrane domains of TANGO1 (Figure S2, top panel, lanes 4 and 6). Extraction with low concentration (0.05%) of Triton X-100 followed by trypsin treatment digested the protein, which was not recognized by both anti-FLAG and anti-HA antibodies (Figure S2, lane 5).

SOSUI and PHDhtm, programs that predict potential TM domains, suggest two such domains at amino acids 1143–1165 and 1183–1205, respectively, in TANGO1. This is intriguing because the results described above (Figures 2A, 2B, S2) suggest that the N-terminal half of TANGO1 is in the lumen and the C-terminal half is in the cytoplasm. To achieve this topology, however, there should be either one or three TM domains. To test this experimentally, we generated recombinant TANGO1-HA (HA tag is at the C terminus) lacking the first (ΔTM1), second (ΔTM2), or both potential TM domains (ΔTM1+2). The respective constructs were expressed in HeLa cells. Cells were treated with digitonin, fixed, and stained

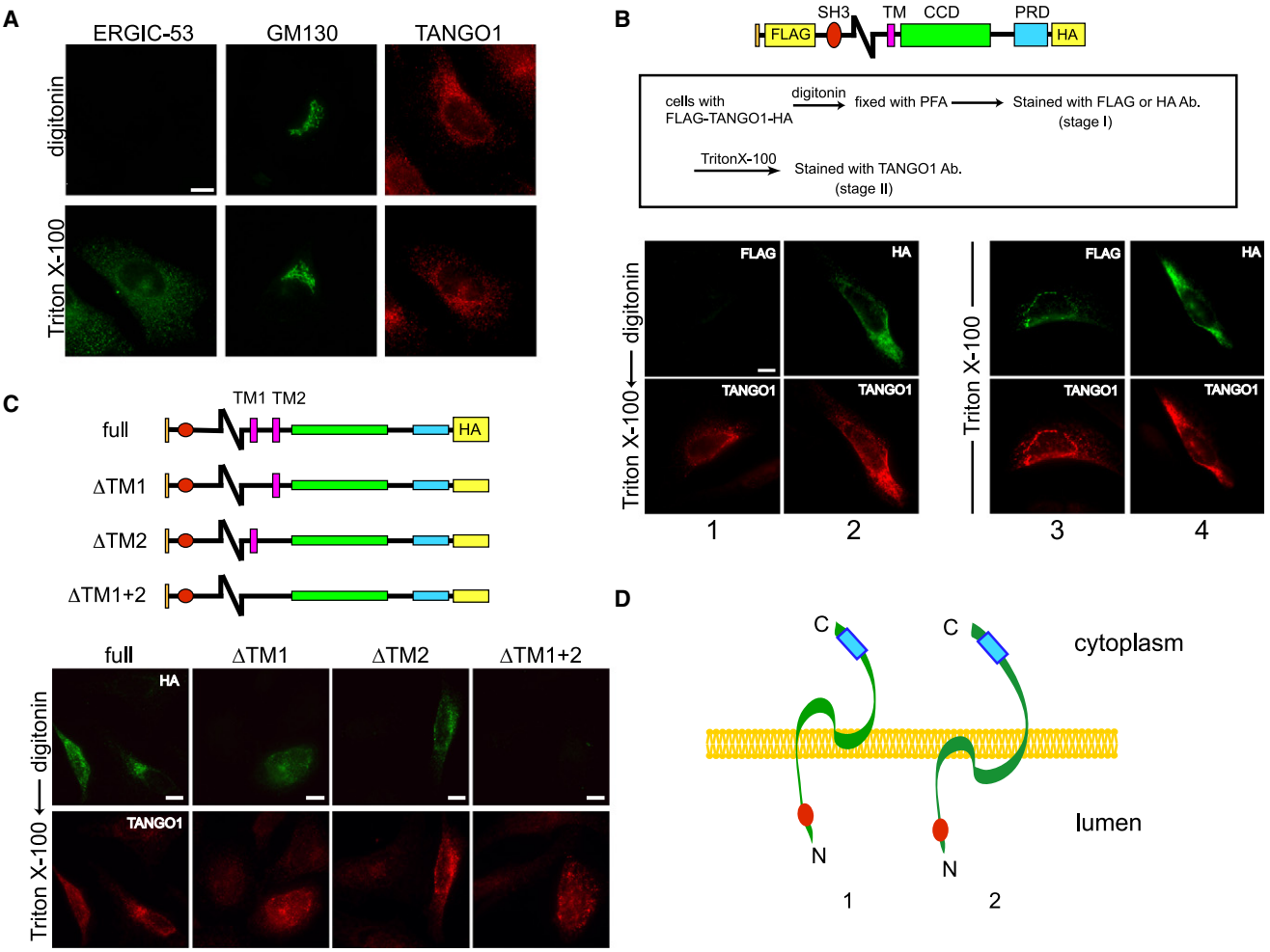

Figure 2. The Topology of TANGO1

(A) (Upper panel) HeLa cells were permeabilized with digitonin, fixed with PFA, and stained with anti-ERGIC-53, anti-GM130, and anti-TANGO1 antibodies. (Lower panel) HeLa cells were fixed with PFA and treated with Triton X-100. The cells were stained with antibodies to ERGIC-53, GM130, and TANGO1. Scale bar, 10 μm.

(B) FLAG-TANGO1-HA was expressed in HeLa cells. The cells were permeabilized with digitonin, fixed with PFA, and incubated with either anti-FLAG (lane 1) or anti-HA (lane 2) antibodies (stage I). The cells were treated with Triton X-100 followed by incubation with anti-TANGO1 (lanes 1 and 2) antibody (stage II). The cells were processed and incubated with secondary antibodies. HeLa cells expressing FLAG-TANGO1-HA were fixed and permeabilized with Triton X-100 and incubated with anti-TANGO1 antibody and either anti-FLAG (lane 3) or anti-HA (lane 4) antibodies, respectively. Scale bar, 10μm.

(C) HA-tagged full-length ΔTM1, ΔTM2, and ΔTM1+TM2 were expressed in HeLa cells. The cells were permeabilized with digitonin, fixed with PFA, and incubated with anti-HA antibodies. The cells were washed and then treated with Triton X-100 and processed for immunofluorescence microscopy with anti-TANGO1 antibody. Scale bar, 10 μm.

(D) Two possible models of the TM domains in TANGO1. In the first, amino acids 1143–1165 span the ER membranes, whereas amino acids 1183–1205 are partially embedded in the outer leaflet. In the second, amino acids 1143–1165 are partially embedded in the luminal leaflet, whereas amino acids 1183–1205 span the ER membranes.

with HA antibody. The cells were then treated with Triton X-100 and stained with TANGO1 antibody. We hypothesized that deletion of all TM domains would cause the polypeptide to be entirely luminal and, therefore, not bound to the C-terminal-specific anti-HA antibody in cells treated with digitonin only. Anti-TANGO1 antibody will recognize both the endogenous and exogenous TANGO1, but the cells expressing the exogenous protein will reveal higher (brighter) staining. As shown in Figure 2C, deletion of either TM domain did not affect staining with the anti-HA antibody. However, deletion of both TM domains resulted in the loss of staining. Based on these data, we suggest that one of the TM domains spans the membrane, whereas the second must be partially inserted into the inner or outer leaflet but does not cross the ER membrane (Figure 2D).

TANGO1 Localizes to ER Exit Sites by Its PRD

Three different mutant forms of TANGO1 were generated and expressed in HeLa cells. Construct ΔSH3 lacks the luminally oriented SH3 domain, amino acids 48–106; construct ΔCT lacks the C-terminal amino acids 1601–1907; and construct ΔCT2

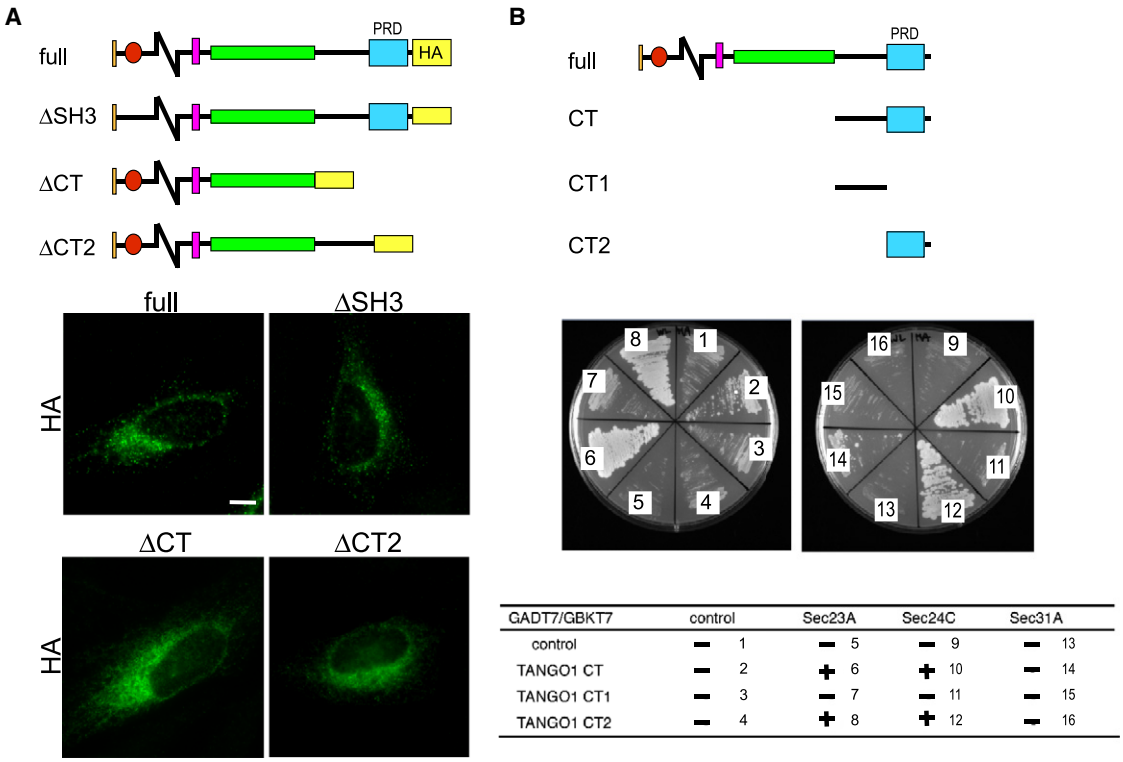

Figure 3. The PRD of TANGO1 Is Required for Its Localization at the ER Exit Sites

(A) HA-tagged full-length TANGO1, ΔSH3-TANGO-HA, ΔCT-TANGO-HA, and ΔCT2-TANGO-HA were expressed in HeLa cells. The cells were fixed with methanol and incubated with anti-HA antibody to visualize the location of the expressed proteins. Loss of C-terminal region post coiled-coiled domain (ΔCT) and PRD (ΔCT2) causes the expressed protein to relocate throughout the ER. Scale bar, 10 μm.

(B) CT, CT1, and CT2 domains of TANGO1 in pGADT7 plasmids were cotransformed with pGBKT7 plasmids containing Sec23A, Sec24C, or Sec31A into AH109 yeast strains and plated on tryptophan- and leucine-deficient medium. Cotransformed cells were replated on tryptophan-, leucine-, histidine-, and adenine-deficient medium. Cell growth was observed in CT and CT2 cotransformed with Sec23A (marked with the numbers 6 and 8) and Sec24C (numbers 10 and 12), respectively.

lacks amino acids 1751–1907. All constructs that contained a C-terminal HA tag were visualized with anti-HA antibody. Construct ΔSH3 localized to the ER exit sites similarly to the full-length protein (Figure 3A, upper panel). Constructs ΔCT and ΔCT2, lacking the PRD, were found dispersed in the ER (Figure 3A, lower panel). This suggests that the PRD is required for the localization of TANGO1 to the ER exit sites.

COPII component Sec31 contains a PRD that binds to Sec23/Sec24 (Bi et al., 2007; Fromme et al., 2007; Shaywitz et al., 1997; Shugrue et al., 1999). We tested by yeast two-hybrid whether the TANGO1 PRD also interacts with Sec23/Sec24. The PRD in TANGO1 stretches from amino acids 1751–1907. Figure 4B shows three constructs of TANGO1 used to investigate the importance of the PRD: CT (amino acids 1601–1907), CT1 (1601–1750), and CT2 (1751–1907) were used in yeast two-hybrid for interaction with COPII components Sec23A, Sec24C, and Sec31A. Only the PRD containing constructs CT and CT2 were found to interact with both Sec23A and Sec24C (Figure 3B). There was no interaction detected between the CT domains and Sec31A (Figure 3B). These results indicate that the C-terminal PRD of TANGO1 can bind to Sec23 and Sec24. Given that this domain is required for ER exit site localization, it seems reasonable to conclude that TANGO1 is localized to ER exit sites by interaction with Sec23/Sec24.

The SH3 Domain of TANGO1 Is Required for Collagen VII Binding

TANGO1 was selected in a screen for genes involved in secretion of the soluble protein ss-horseradish peroxidase (HRP) in *Drosophila* tissue culture S2 cells (Bard et al., 2006). HeLa cells were transfected with control or TANGO1 siRNA followed by transfection with ss-HRP. Knockdown of TANGO1 inhibited HRP secretion by about 50% (Figure S3). However, TANGO1 depletion by siRNA in HeLa cells did not affect the trafficking of another secreted protein, alkaline phosphatase (Figure S3), and the integral membrane protein, VSV-G (data not shown). The effect of TANGO1 depletion on the secretion of exogenously expressed, plant-specific protein HRP is, therefore, quite surprising.

To further test the involvement of TANGO1 in protein secretion and cellular physiology, we searched for TANGO1 binding partners by mass spectrometric analysis of immunoprecipitated endogenous TANGO1 from HeLa cell extracts. This procedure revealed six specific peptides of human collagen VII

A

aa.	collagen VII peptide
59-78	SFLEGLVLPFSGAASAQGVR
90-105	TEFGLDALGSGGDVIR
728-754	SQLVSGEATVAELDGLEPDTEYTVHVR
852-878	EGTPVSIVVTTPPEAPPALGTLHVVQR
1015-1036	VTGLEPGVSYIFSLTPVLDGVR
1958-1976	EIVETWDESSGSFLPVPER

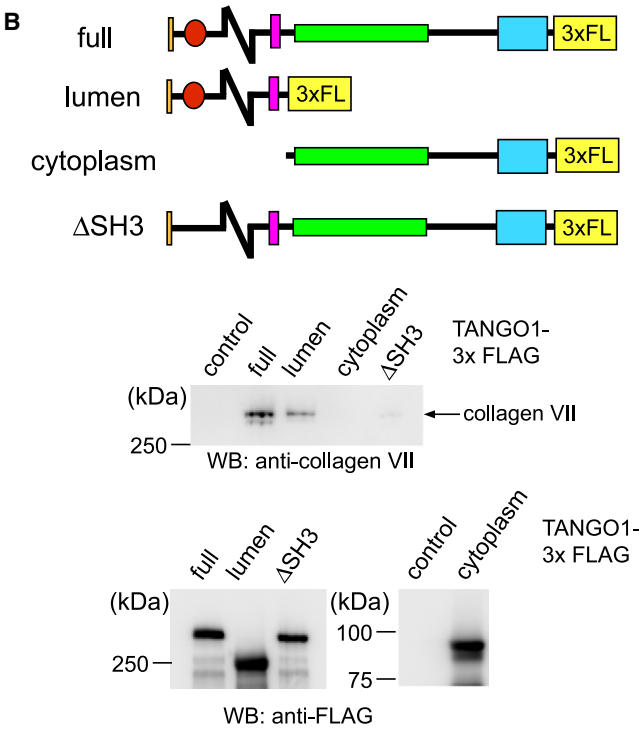

Figure 4. TANGO1 Binds to Collagen VII

(A) HeLa cell extracts were immunoprecipitated with anti-TANGO1 antibody, and the bound polypeptides were eluted with TANGO1 peptide used for generating the antibody and then sequenced by mass spectrometry. Six peptides corresponding to collagen VII were contained in the immunoprecipitant with anti-TANGO1 antibody.

(B) The C-terminal 3X FLAG-tagged full-length (full), luminal (lumen), cytoplasmic (cytoplasm), and full-length lacking SH3 domain (ΔSH3) were expressed in COS7 cells (top panel). The respective proteins were immunoprecipitated with anti-FLAG agarose beads and incubated with culture medium from RDEB/FB/C7 cells containing secreted collagen VII. The beads were washed and analyzed by SDS-PAGE followed by western blotting with anti-collagen VII antibody (middle panel) and anti-FLAG antibody (lower panel).

(Figure 4A). Since collagen VII is a secreted protein, it is likely to bind to the luminal domain of TANGO1. To test this proposal, we generated various domains of FLAG-tagged TANGO1. These domains were expressed in COS7 cells and immunoisolated with anti-FLAG antibody. Tissue culture medium containing secreted collagen VII from RDEB/FB/C7 cells was collected and incubated with beads containing immunoisolated FLAG-TANGO1. The beads were washed and subjected to SDS-PAGE followed by western blotting with anti-collagen VII antibody. The results show that full-length FLAG-TANGO1 (full) and the luminal domain of TANGO1 (lumen) bind to collagen VII (Figure 4B). TANGO1 lacking the luminal domain (cytoplasm) or the SH3 domain (ΔSH3) does not bind to collagen VII (Figure 4B). The same results were obtained when recombinant proteins were used as a source for collagen VII (data not shown). These findings strongly indicate the significance of the luminal SH3 domain of TANGO1 for binding to collagen VII.

TANGO1 Is Required for Collagen VII Secretion

Collagen VII is expressed in skin keratinocytes and fibroblasts. Therefore, we tested these cell types for the significance of TANGO1 in collagen VII secretion. We first tested A431 (human keratinocyte-like) cells for a role of TANGO1 in collagen VII secretion. Although these cells synthesize and secrete collagen VII, it is essentially impossible to detect an intracellular pool of collagen VII by immunofluorescence microscopy under steady-state conditions. However, about 50% of the cells revealed accumulation of collagen VII in the ER upon depletion of TANGO1 by siRNA (Figure 5A). This suggests that, without TANGO1, synthesized collagen VII fails to exit the ER. Collagen VII, consequently, accumulates in the ER and is, therefore, detected by immunofluorescence microscopy.

To rule out the possibility that TANGO1 transcriptionally regulates collagen VII synthesis, we tested human 293/C7 cells stably expressing collagen VII under cytomegalovirus (CMV) promoter for the involvement of TANGO1 in its export. The 293/C7 cells were transfected with control or TANGO1 siRNA. Culture medium from the cells was western blotted with anti-collagen VII antibody. The results revealed that the quantity of collagen VII secreted from TANGO1-depleted cells was about 80% less compared with control cells (Figure 5B). There was a concomitant, 3-fold increase in the intracellular pool of collagen VII in cells depleted of TANGO1 (Figure 5B). We then tested the involvement of TANGO1 in collagen VII secretion from RDEB/FB/C7 human skin fibroblast cells. These fibroblasts stably express and secrete collagen VII and also secrete a detectable amount of endogenous collagen I. Depletion of TANGO1 revealed > 70% inhibition in collagen VII secretion without any appreciable effect on collagen I secretion (Figure 5C). To further ascertain the involvement of TANGO1 in collagen I secretion, we transfected human fibroblast cell line Saos2, which synthesizes and secretes collagen I, with control or TANGO1 siRNA. These cells do not secrete collagen VII. TANGO1 was efficiently depleted from these cells (> 90% knockdown), but there were no obvious effects on the secretion of collagen I (Figure 5D).

Ascorbic acid, a cofactor for prolyl 4-hydroxylase, regulates synthesis and folding of collagens to generate a trimeric collagen in the ER (Pinnel et al., 1987). We tested the effect of ascorbic acid on collagen VII secretion in RDEB/FB/C7 cells. Because these cells express collagen VII under a CMV promoter, the effects of ascorbic acid on the synthesis of collagen VII can be excluded. The addition of 2 μg/ml ascorbic acid to the cells caused about a 5-fold increase in collagen VII secretion (Figure S4). RDEB/FB/C7 cells grown in the presence of ascorbic acid were transfected with TANGO1 siRNA, and its effects on collagen VII secretion were monitored by western blotting. The

secreted collagen was analyzed in the absence or presence of dithiothreitol (DTT) to visualize trimeric (900 kDa) and monomeric (290 kDa) collagen VII. Our findings reveal that RDEB/FB/C7 cells secrete trimeric collagen, which increases in the presence of ascorbic acid, and that knockdown of TANGO1 inhibited secretion of trimeric collagen VII by > 50%. Under the same conditions, collagen I secretion was not affected (data not shown). These results strongly indicate that TANGO1 is required for the secretion of fully folded trimeric collagen VII (Figure S4).

To test whether TANGO1 is required for general protein export from the ER, A431 cells were transfected with control or TANGO1 siRNA. The cells were pulsed with [^{35}S]-methionine for 1 hr followed by a chase for 3 hr. The cells treated with Brefeldin A (BFA), an inhibitor of protein export, were also pulsed and chased. The medium was collected at 0, 1, 2, and 3 hr of chase and analyzed by SDS-PAGE on 6% and 12% gels (to separate the high- and low-molecular weight polypeptides) followed by autoradiography. Our findings reveal that TANGO1 knockdown does not affect the overall amount of protein secreted by A431 cells (Figure 5E). BFA treatment of the control cells, as expected, efficiently inhibited the amount of total protein secretion from A431 cells (Figure 5E).

We also tested the effect of TANGO1 knockdown on the organization of ER and the ER exit sites. We observed an aggregation of Sec16L and Sec24D upon TANGO1 knockdown in A431 cells (Figure S5). Electron microscopy analysis revealed a 2-fold increase in the average length of ER cisternae and a 2.5-fold increase in the density of ER in TANGO1-depleted A431 cells (Figure S6). HeLa cells, which synthesize and secrete less collagen VII than do A431 cells, showed marginal effects on the organization of Sec16L, Sec24D, and the overall ER organization (data not shown). Thus, the effects of TANGO1 in the organization of ER exit sites and ER are likely indirect due to the accumulation of collagen VII within the ER.

Taken together, our findings suggest that TANGO1 does not have a role in global protein secretion; it appears to be specific for secretion of trimeric collagen VII.

TANGO1 Does Not Exit the ER

TANGO1 binds to collagen VII in the lumen of the ER and COPII components, Sec23/24 on the cytoplasmic side. These interactions require SH3 and PRD of TANGO1, respectively. Is TANGO1 loaded into COPII carriers and exported from the ER? This is an important issue as other cargo receptors such as ERGIC53, Erv29p, and KDEL-R bind their specific cargoes and are exported from the budding compartment. We used an in vitro COPII budding assay to test the hypothesis of whether TANGO1 is exported in COPII carriers from the ER. Permeabilized HeLa cells were incubated with rat liver cytosol, ATP-regenerating system, and GTP for 1 hr at 30°C, and the budded vesicles were collected as described previously (Fromme et al., 2007; Kim et al., 2007a, 2007b). As expected, the anterograde cargoes, ERGIC53 and APP, were contained in these carriers (Figure 6, lanes 5 and 7). Incubation with GTPγS instead of GTP inhibited this reaction as described previously (Figure 6, lane 9) (Fromme et al., 2007; Kim et al., 2007a, 2007b). COPII carrier formation is also inhibited by incubation with the GTP-restricted form of Sar1, Sar1H79G (Figure 6, lane 6), but not with preinactivated Sar1H79G (Figure 6, lane 7). Interestingly, like Ribophorin I, which is a resident protein of the ER, TANGO1 was excluded from the COPII carriers (Figure 6, lanes 5 and 7). These results indicate that, although TANGO1 binds to the cargo and COPII coats, it is not incorporated into COPII carriers at the ER exit sites.

DISCUSSION

We have described here the role of TANGO1, a protein that loads collagen VII into transport carriers without following the cargo into the vesicle itself. Thus, the mechanism of TANGO1-mediated cargo loading is distinct from other previously described cargo receptors, and this makes it unique among the known guides of protein secretion. Collagen VII is a bulky 900 kDa trimer that is too big to fit into a generic COPII vesicle of 60–90 nm diameter. Therefore, TANGO1 provides a means to begin to address the mechanism regulating the size of transport carriers; moreover, upon secretion, collagen VII interacts with other adhesive proteins and helps in the attachment of epidermis to the dermis (Chen et al., 2002). Patients with genetic defects in collagen VII or with autoantibodies to collagen VII have a number of severe skin disorders, which are collectively called dystrophic epidermolysis bullosa (DEB) and epidermolysis bullosa acquisita (EBA), respectively (Chen et al., 2002; Woodley et al., 2007). TANGO1-mediated collagen VII export could reveal important insights into the process of skin biogenesis.

Until recently, there was a strong bias toward the proposal that cargo export from the ER is by bulk flow (Wieland et al., 1987). However, the identification of proteins such as ERGIC53, Erv29p/Surf4, and Emp24p has shifted the balance in support of the proposal that cargo export from the ER is receptor mediated. Identification of TANGO1 as a guide for secretory cargo such as collagen VII provides further support to the latter proposal.

TANGO1 Is Distinct from Other Guides for Cargo Loading

There are at least 30 different kinds of vertebrate-specific collagens and numerous collagen-like molecules that are required for tissue assembly or maintenance (Kadler et al., 2007). The fact that TANGO1 depletion inhibits secretion of trimeric (900 kDa) collagen VII without affecting transport of collagen I indicates that TANGO1 is an unlikely candidate for the general synthesis, posttranslational modification, and enzymatic folding of collagens. TANGO1, therefore, loads folded trimeric collagen VII into the newly forming COPII transport carriers. During this process, it remains anchored at ER exit sites. TANGO1 is, therefore, distinct from other membrane proteins involved in cargo loading with respect to its choice of cargo and location during protein export from the ER.

TANGO1 May Slow COPII Biogenesis until Collagen VII Is Loaded

TANGO1 has a number of unique features that make it an attractive candidate for cargo loading. It contains an SH3-like domain in the lumen of the ER and a PRD facing the cytoplasm. This arrangement allows TANGO1 at the site of COPII carrier

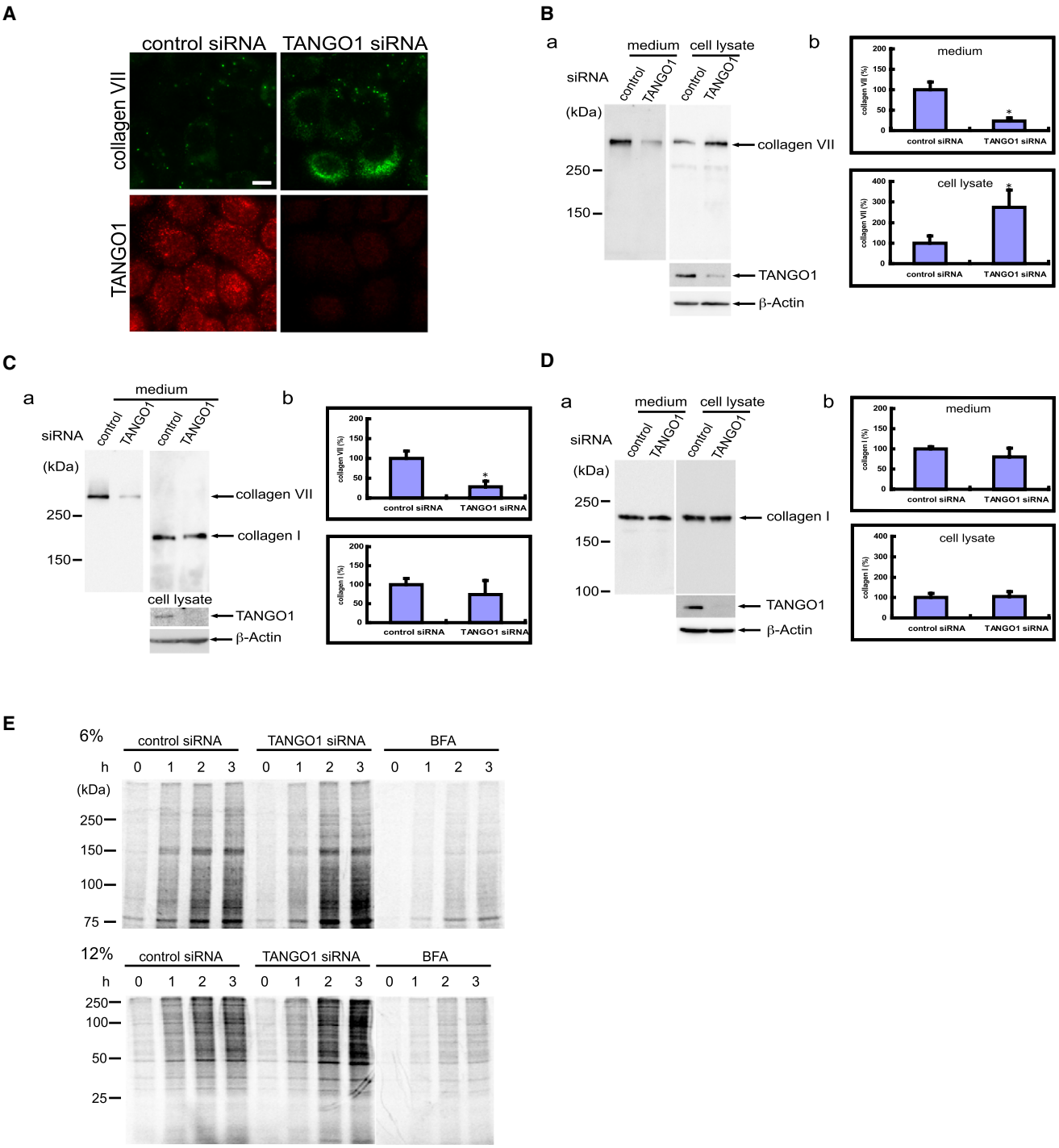

Figure 5. TANGO1 Is Required for Collagen VII Export from the ER

(A) A431 cells that synthesize and secrete endogenous collagen VII were transfected with control or TANGO1 siRNA. The cells were fixed and stained with anti-collagen VII and anti-TANGO1 antibodies. Knockdown of TANGO1 caused accumulation of collagen VII in the ER in about 50% of cells. Scale bar, 10 μm.

(B) The 293/C7 cells stably expressing collagen VII were transfected with control or TANGO1 siRNA. (a) The medium was western blotted with anti-collagen VII antibody. The lysates were western blotted with anti-collagen VII, TANGO1, and β-Actin antibodies. (b) An average of three different experiments revealed that knockdown of TANGO1 caused an 80% reduction in collagen VII secretion and a 3-fold increase in the intracellular pool of collagen VII. Error bars represent mean ± SD. *$p < 0.05$ compared with the control siRNA.

(C) A stable cell line RDEB/FB/C7 that synthesizes and secretes exogenously expressed collagen VII and endogenous collagen I was transfected with control or TANGO1 siRNA. (a) The medium was western blotted with anti-collagen VII and collagen I antibodies, respectively. The lysates were western blotted with

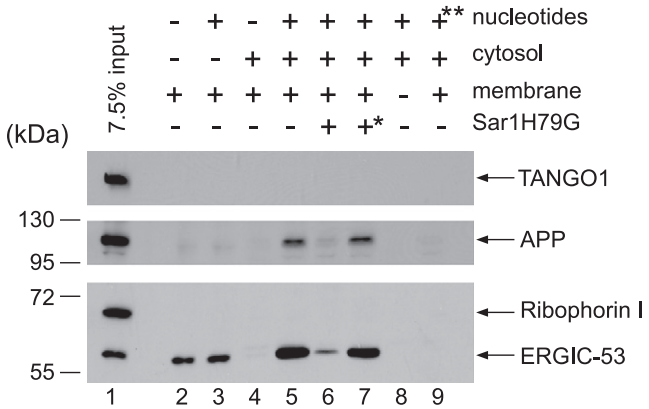

Figure 6. TANGO1 Is Not Loaded into COPII Carriers

Digitonin-permeabilized HeLa cells (donor membrane) were incubated with ATP regenerating system (lanes 3 and 5–9), GTP (lanes 3 and 5–8), GTPγS (**, lane 9), Sar1 H79G (lane 6), Sar1 H79G pretreated at 65°C for 10 min (*, lane 7), and rat liver cytosol (lanes 4–9). COPII vesicles were collected by centrifugation and western blotted with anti-TANGO1, anti-amyloid precursor protein (APP), anti-ribophorin I, and anti-ERGIC53 antibodies. (Lane 1) Input, 7.5% of the donor membrane.

biogenesis to bind cargo by the luminal SH3 domain and COPII components Sec23/24 through its cytoplasmic PRD. Interestingly, both TANGO1 and Sec31 contain a PRD, which interacts with Sec23/24 of COPII coats. The binding of TANGO1's PRD could affect recruitment of Sec13/31 to Sec23/24 and thus stall the completion of COPII biogenesis. Accordingly, when the PRD is bound to Sec23/24, the COPII carrier grows as the SH3-bound collagen VII is guided from the luminal side. Once cargo has been loaded, a change in the conformation of TANGO1 could induce dissociation of the PRD from Sec23/24 and collagen VII from the SH3 domain, which could trigger the separation of a cargo-filled COPII carrier. TANGO1 has a single transmembrane domain and an additional hydrophobic stretch, which we suggest partially spans the membrane, as found in another ER resident protein, Rtn4a (Voeltz et al., 2006). While the transmembrane domain anchors TANGO1 in the membrane, the partially embedded hydrophobic domain might act as a pivot to move the cytoplasmic domain (~1000 amino acid), thus regulating the dynamics of TANGO1 in a cargo-dependent manner (Figure 7). This arrangement allows TANGO1 to load cargo without entering the newly forming transport carrier.

Is TANGO1 Specific for Collagen VII Export?

All human cells, except those of hematopoietic origin, express TANGO1 mRNA (Bosserhoff et al., 2004). Collagen VII, however, is secreted predominantly by epidermal keratinocytes and dermal fibroblasts (Chen et al., 1994). Because TANGO1 is expressed only in cells that attach directly or indirectly by an extra cellular matrix (ECM), it is reasonable to propose that, in cells that do not secrete collagen VII, TANGO1 exports different secretory cargo, which might also be involved in cell-cell attachment. Yet, why does TANGO1 depletion inhibit secretion of the exogenously expressed plant protein HRP by about 50% (albeit not as strongly as collagen VII) (Bard et al., 2006)? There is no obvious sequence homology between HRP and collagen VII. It is, therefore, difficult to imagine that TANGO1 recognizes a common sequence between these two proteins. We hope that identification of other cargoes exported by TANGO1 will reveal whether the affects on HRP secretion are direct or whether HRP has features that make it a weak binding partner of TANGO1.

The Effect of TANGO1 Depletion on the ER and ER Exit Sites

Knockdown of TANGO1 in A431 cells caused aggregation of Sec16L and Sec24D, and the ER increased in length and density (Figures S5 and S6). HeLa cells, which secrete less collagen VII than do A431 cells, revealed very minor changes in the organization of Sec16L, Sec24D, and the overall ER (data not shown). The effects of TANGO1 knockdown on ER organization and exit sites are most likely indirect due to accumulation of collagen VII. It has recently been reported that knockdown of Sec13 also causes aggregation of Sec24C and Sec16 in HeLa cells (Townley et al., 2008). Interestingly, although transport of VSV-G protein to the cell surface was largely unperturbed, export of procollagen I from the ER was blocked under such conditions. The effect on the organization of ER exit sites upon Sec13 knockdown, we suggest, is indirect as well, due to the accumulation of procollagen I in the ER. These findings, however, raise an important issue. If clustering of ER exit sites does not affect general protein secretion, why are they distributed throughout the ER?

In sum, TANGO1 employs a unique mechanism to load secretory cargo into COPII carriers. Identification of other TANGO1-like cargo guides and cargoes exported by TANGO1 will help in understanding the mechanism of protein export from the ER, which is diverse in quality and large in quantity.

EXPERIMENTAL PROCEDURES

Antibodies

TANGO1 polyclonal antibody was raised in rabbits by immunization with keyhole limpet hemocyanin-conjugated peptide (C-SRDEPPPASQSTSQD) corresponding to the C-terminal amino acids 1884–1898 of TANGO1. The

anti-TANGO1 and β-Actin antibodies. (b) Quantitation of three different experiments revealed > 70% decrease in collagen VII secretion. Collagen I secretion, on the other hand, was not significantly affected by TANGO1 depletion. Error bars represent mean ± SD. *$p < 0.01$ compared with the control siRNA.

(D) Saos2 cells that synthesize and secrete endogenous collagen I were incubated with control or TANGO1 siRNA. (a) The medium was western blotted with anti-collagen I antibody; the cell lysates were western blotted with anti-collagen I, TANGO1, and β-Actin antibodies, respectively. (b) Quantitation of three different experiments revealed statistically no effects on collagen I secretion upon TANGO1 depletion compared with control cells. Error bars represent mean ± SD.

(E) A431 cells were transfected with control or TANGO1 siRNA. After 48 hr, the cells were labeled with [^{35}S]-methionine for 1 hr. The cells were washed and then cultured in standard medium containing unlabeled methionine. At 0, 1, 2, and 3 hr after the chase, the medium was analyzed by SDS-PAGE on 6% (top) and 12% (bottom) gels followed by autoradiography. Brefeldin A (BFA) was added to one-half of the control cells for the last 10 min of incubation with [^{35}S]-methionine and kept throughout the chase. Treatment with BFA inhibited global protein secretion, whereas TANGO1 knockdown did not reveal an appreciable reduction in the total amount of proteins secreted.

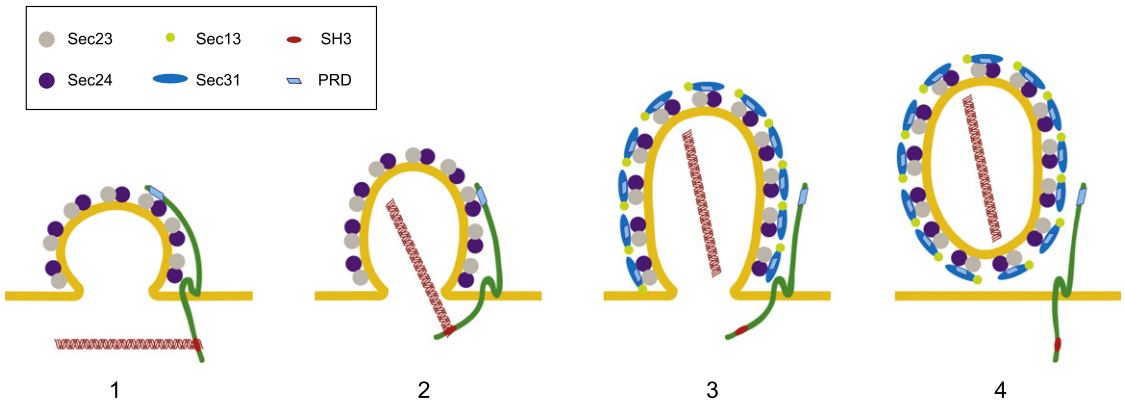

Figure 7. A Model for TANGO1-Mediated Cargo Loading into COPII Carriers

TANGO1 is anchored to the ER exit site. We propose that, when secretory cargo such as collagen VII is synthesized, it binds to TANGO1's SH3 domain, which is accompanied by the binding of PRD to Sec23/24 subunits of COPII coats (1). During this period, the cargo is pushed into the growing COPII carrier (2). Once fully loaded with the cargo, the SH3 and PRD dissociate from their respective partners (3). This allows recruitment of Sec13/31 to finish the biogenesis of a cargo-filled carrier (4).

antibody was affinity purified on peptide column (Pacific Immunology, USA). Collagen VII polyclonal antibody to NC2 domain was used for western blot as described previously (Chen et al., 1997). Antibodies to Sec24D, APP, and Ribophorin I were used as described previously (Fromme et al., 2007; Kim et al., 2007a, 2007b). Antibodies to Sec31A, GM130, and Collagen VII α1 were purchased from BD Biosciences. Collagen I monoclonal antibody (SP1.D8) was obtained from the Developmental Studies Hybridoma Bank. Other antibodies were purchased from the following companies: ERGIC-53 (Alexis), Sec16L (KIAA0310 Bethyl), β-COP (Abcam), β-Actin (SIGMA), FLAG M2 (SIGMA), and HA (Covance). Secondary antibodies for immunofluorescence microscopy were from Molecular Probes.

Immunofluorescence Microscopy

Cells grown on coverslips were fixed with cold methanol for 6 min unless otherwise specified. Cells were blocked with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 for 15 min. The primary antibody was incubated for 1 hr at room temperature, and secondary antibodies conjugated with Alexa 488 or Alexa 594 were used for detection. Images were taken with a CCD camera (photometric cool snap HQ) mounted on a Nikon eclipse TE2000-U. Images were deconvolved with Autoquant when specified.

TANGO1 Topology by Immunofluorescence Microscopy

HeLa cells on coverslips were washed with KHM buffer consisting of 20 mM HEPES (pH 7.4), 110 mM potassium acetate, and 2 mM magnesium acetate and then treated with KHM buffer containing 25 μg/ml digitonin for 5 min on ice. Cells were washed once with KHM buffer and fixed with 4% PFA in PBS for 10 min at room temperature. Cells were blocked with PBS containing 5% BSA for 15 min and incubated with primary antibody for 1 hr. After washing three times with PBS, cells were permeabilized with PBS containing 5% BSA and 0.1% Triton X-100 for 15 min. The cells were incubated with the other primary antibody and secondary antibodies as described above.

Yeast Two-Hybrid Assay

Matchmaker yeast two-hybrid system 3 (Clontech) was used. AH109 strain was transformed with pGBKT7 and pGADT7 vectors and plated on tryptophan- and leucine-deficient medium. The transformed colonies were further plated on tryptophan-, leucine-, histidine-, and adenine-deficient plates for interaction assay.

Mass Spectrometry Analysis

HeLa cells were extracted with extraction buffer consisting of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors and incubated for 30 min at 4°C. The lysate was centrifuged at 20,000 × g for 20 min. The supernatants were incubated with TANGO1 antibody with protein A beads for 2 hr at 4°C. As for control, TANGO1 antibody was preincubated with more than 100-fold excess of TANGO1 peptide used for generating antibody for 2 hr at 4°C before being added to the supernatants. The beads were washed with TBS/0.1% Triton X-100 and eluted with TANGO1 peptide. A fraction of eluted materials was analyzed by SDS-PAGE followed by silver staining. The control eluate gave no detectable bands. The peptide was removed by iodoacetamide precipitation. The eluted materials were digested with trypsin, and the resulting peptides were analyzed by microcapillary liquid chromatography and tandem mass spectrometry (MS). An in-house MS system consisting of an HPLC and LTQ ion trap MS (Thermo Finnigan) was used. The SEQUEST software package was used to analyze tandem mass spectra using a mouse ORF database with no restriction on the protease used. Only tryptic peptides identified after database searches were further manually inspected for correct identification of the expected peptide fragments in the tandem mass spectra.

In Vitro Binding

COS7 cells were transfected with 3X FLAG alone, TANGO1 full-length 3X FLAG, TANGO1 lumen (amino acids 1–1210) 3X FLAG, TANGO1 cytoplasm (amino acids 1211–1907) 3X FLAG, and TANGO1 ΔSH3 3X FLAG and cultured for 48 hr. Cells were extracted with extraction buffer and incubated for 15 min at 4°C. The lysates were centrifuged at 20,000 × g for 20 min. The supernatants were incubated with anti-FLAG M2 agarose beads (SIGMA) for 2 hr at 4°C. The beads were washed with TBS/0.1% Triton X-100 and subsequently incubated with culture medium from RDEB/FB/C7 cells (cultured for 48 hr in the presence of 2 μg/ml ascorbic acid) for 2 hr at 4°C. The beads were washed with TBS/0.1% Triton X-100 and prepared for SDS-PAGE analysis.

Collagen Secretion

The 293/C7 (for collagen VII), RDEB/FB/C7 (for collagen VII and collagen I), and Saos2 (for collagen I) cells were transfected with siRNA and cultured in the presence (2 μg/ml ascorbic acid) or absence of ascorbic acid. At 24 hr after siRNA transfection, cells were washed with PBS, and the mediums were replaced and further cultured for 24–48 hr in the presence or absence of ascorbic acid. The mediums and cells were collected separately. The mediums were centrifuged briefly and boiled for 5 min with Laemmli sample buffer with or without DTT and subjected to SDS-PAGE for analyzing the presence of collagens. The mediums were also western blotted with β-actin to verify that there was no contamination of cell debris. The cells were washed with PBS, extracted, and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were boiled for 5 min with Laemmli sample buffer with DTT and subjected to

SDS-PAGE and western blotted with anti-collagens, TANGO1, and β-actin antibodies. Image J (NIH, USA) was used for quantitation. The signals were normalized by β-actin.

Metabolic Labeling

Control or TANGO1 siRNA-treated or untreated A431 cells were cultured in a 6-well dish with DMEM without L-methionine and L-cysteine for 4 hr and then pulsed with 50 μCi of [^{35}S] methionine (Perkin Elmer) for 1 hr. Cells were washed for five times with PBS and then chased with DMEM containing 10 mM L-methionine. The medium was collected at 0, 1, 2, and 3 hr. For BFA treatment, 10 μg/ml of BFA was added to the medium for the last 10 min of pulse and kept for whole-chase experiments. After 3 hr of chase, the cells were washed with PBS for five times and collected. The cells were extracted with 1 ml of TBS/1% Triton X-100. Then, 20 μl of extracts were mixed with scintillation cocktail, and the radioactivity was measured by scintillation counter for normalization. The medium collected was analyzed by SDS-PAGE followed by autoradiography.

In Vitro Vesicle Formation Assay

The assay was essentially performed as described previously (Fromme et al., 2007; Kim et al., 2007a, 2007b). Donor membranes were prepared from HeLa cells by permeabilizing with digitonin. Rat liver cytosol was prepared as described previously (Fromme et al., 2007; Kim et al., 2007a, 2007b). The donor membranes were incubated at 30°C for 1 hr in 20 mM HEPES-KOH (pH 7.2), 250 mM Sorbitol, and 150 mM KOAc supplemented with ATP regenerating system (1 mM ATP, 40 mM creatine phosphate, and 0.2 mg/ml creatine phosphokinase), 0.3 mM GTP or GTPγS, 4 μg/ml Sar1H79G, and 4.8 mg/ml rat liver cytosol. The reaction mixtures were centrifuged at 13,000 × g for 12 min at 4°C, and the supernatants were subsequently centrifuged at 55,000 rpm for 25 min at 4°C. The precipitants were resuspended and analyzed by SDS-PAGE and western blotting.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01630-9.

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